Bacillus subtilis spoVE Gene Is Transcribed by \( \sigma^E \)-Associated RNA Polymerase

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Expression of the Bacillus subtilis sporulation gene spoVE was examined by runoff transcription assay with an RNA polymerase preparation obtained from vegetative and sporulating cells. Transcripts from tandem promoters (P1 and P2 promoters) located just upstream of the spoVE structure gene were detected. The transcription of spoVE initiated within an hour after the onset of sporulation and coincided with the presence of RNA polymerase associated with a 33-kDa protein. Amino acid sequence analysis of the 33-kDa protein revealed that it is a sigma factor, \( \sigma^E \). Reconstitution analysis of \( \sigma^E \)-purified from the sporulating cell extract and vegetative core RNA polymerase showed that \( \sigma^E \) recognizes the P2 promoter. SpoVE protein could not be synthesized in the transcription-translation coupled system prepared from vegetative cells (M. Okamoto, S. Fukui, and Y. Kobayashi, Agric. Biol. Chem. 49:1077–1082, 1985). However, addition of \( \sigma^E \)-associated RNA polymerase to the coupled system restored SpoVE protein synthesis. These results indicate that spoVE expression in sporulating cells is controlled essentially by \( \sigma^E \)-associated RNA polymerase.

In a nutrient-rich condition, Bacillus subtilis cells grow and divide symmetrically. When the environment becomes deficient in nutrients, cells form an asymmetric septum and begin spor formation. Sporulation of B. subtilis is regarded as a simple form of cellular differentiation. It is known that approximately 50 genetic loci are required for this process (15).

A spoVE mutation blocks sporulation at stage V, and the mutant cannot synthesize cortex but can synthesize spore coat (25). The spoVE gene has been cloned in a 2.8-kb EcoRI fragment (30), and the nucleotide sequence was determined (11, 27). S1 nuclease analysis and spoVE-subtilis gene fusion analysis showed that two promoters for spoVE transcription exist just upstream of the spoVE structural gene (29). Ikeda et al. (8) indicated that the amino acid sequence of SpoVE is homologous to that of Escherichia coli FtsW and RodA, which participate in septum formation and cell elongation, respectively. This fact suggests that the spoVE gene product functions in cortex formation.

Recently, Beall and Luktenhausen (1) determined the nucleotide sequence of a cell division gene cluster which is located downstream of the spoVE gene and may continue upstream of the spoVE gene. It is very interesting that the sporulation gene, spoVE, is present in the cell division gene cluster, gene products of which are required for vegetative growth and cell division. The present paper describes the in vitro expression of spoVE during sporulation and shows that the spoVE gene is transcribed by \( \sigma^E \)-associated RNA polymerase, which is a key enzyme in the early stage of sporulation gene expression.

MATERIALS AND METHODS

Bacterial strains and plasmids. B. subtilis 168WT (spo+) is a laboratory stock. UOT0999 (his101 leuA8 aprE18 nprR2 aprEΔ3) was obtained from the University of Tokyo. JH642 (trpC2 pheA1) was obtained from J. A. Hoch. Plasmid pUCVE-1 is a derivative of pUC18 which has a 2.8-kb EcoRI insert containing the spoVE gene at the multiple cloning site (24). Plasmid pKM-1 is a derivative of pUB110 and pUC18 (31). This plasmid is a shuttle vector between B. subtilis and E. coli.

Small-scale isolation of RNA polymerase. The rapid small-scale method of Gross et al. (4) was used. Cells (UOT0999) were cultivated at 37°C in Schaeffer sporulation medium (28). Vegetative and \( T_0 \) (\( T_0 \) indicates \( n \) h after the initiation of sporulation) cells were harvested from 2-liter cultures, and sporulating cells (1 liter each) were harvested at 1-h intervals (\( T_1 \) to \( T_6 \)). Vegetative and \( T_6 \) cells were washed twice with buffer I (10 mM Tris hydrochloride [pH 7.5], 10 mM MgCl₂, 10% glycerol, 1 M KCl, 10 mM 2-mercaptoethanol, 5 mM Mg-EDTA, and 2 mM phenylmethylsulfonyl fluoride) and then washed once with buffer II, which is the same as washing buffer I except that it contains 50 mM KCl. Sporulating cells were washed four times with buffer I and then washed once with buffer II (20). Washed cells were disrupted with quartz sand for 5 min and extracted with 10 ml of buffer III (50 mM Tris hydrochloride [pH 7.9], 1 mM EDTA, 10% glycerol, 0.5 M NaCl, 5 mM 2-mercaptoethanol, and 2 mM phenylmethylsulfonyl fluoride). After centrifugation, 1 g of polyethylene glycol (PEG) 6000 was added to the supernatant to precipitate DNA bound with RNA polymerase. After centrifugation, RNA polymerase was eluted from the precipitate with 1 ml of buffer IV, which is the same as buffer III except that it contains 2 M NaCl and 10% PEG 6000. The eluate was diluted 10-fold with buffer V (20 mM Tris hydrochloride [pH 7.9], 0.1 mM EDTA, 10% glycerol, and 5 mM 2-mercaptoethanol) and then loaded on a DNA-cellulose column (1.5 ml) which was prepared by Litman’s method (14). The column was washed with 5 ml of buffer V containing 0.15 M NaCl, and then RNA polymerase was eluted with 1.5 ml of buffer V containing 0.75 M NaCl. The eluate was diluted 10-fold with buffer V and then subjected to a DEAE-Cellulose (1 ml; Cisso Co., Ltd) column. The column was washed with 5 ml of buffer V containing 0.11 M NaCl, and then RNA polymerase was eluted with 1.5 ml of buffer V.
containing 0.5 M NaCl. A part of the eluate (0.5 ml) was concentrated by trichloroacetic acid precipitation and then analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide (12.5% acrylamide) gel electrophoresis (see Fig. 2A) by the method of Laemmli and Favre (13). RNA polymerase activity of each eluate prepared from the cells of various stages was measured by the runoff transcription assay with 10 μl each of the eluate (see Fig. 2B). The template of the runoff assay was a 1.3-kb EcoRI-NruI fragment containing spoVE promoters (see Fig. 1). Transcripts were separated by 5% polyacrylamide (containing 7 M urea) gel electrophoresis and detected by autoradiography.

Large-scale isolation of RNA polymerase. The large-scale method of Burgess and Jendrisak (2) was used with some modification. Fifty grams of sporulating cells (168WT) at $T_3$ was harvested from 16 liters of Schaeffer medium with 250 μl of pellet as described above. Fifty were pooled and lyophilized. The concentrated sample was dissolved in 60 ml of buffer IV. After centrifugation, the supernatant was fractionated with ammonium sulfate. Fractions of 30 to 70% saturation were collected, dissolved in 10 ml of buffer V containing 0.5 M NaCl, loaded on a Toyopearl HW65F (Toyo Soda Co., Ltd.) column (2.5 by 100 cm), and then eluted with the same buffer. After gel filtration, RNA polymerase-rich fractions were pooled and dialyzed against buffer V. The diluted fraction was applied on a DNA-cellulose column (2 by 15 cm). RNA polymerase was eluted with an 80-ml linear gradient of NaCl (0.25 to 1.5 M) in buffer V. Fractions (3 ml each) were collected, and the RNA polymerase activity of each fraction was assayed by runoff transcription with 10 μl of each fraction.

Microsequencing of the 33-kDa protein ($\sigma^5$). The RNA polymerase fraction was concentrated with solid PEG 20,000. The concentrated sample was separated by SDS-polyacrylamide (12.5% acrylamide) gel electrophoresis, blotted onto polyvinylidene difluoride paper (Immobilon, Millipore Co.), and then stained with 0.1% amido black 10B (16). The 33-kDa protein band was cut out and subjected to amino acid sequence analysis with the Applied Biosystems model 477A gas-phase sequencer.

Reconstitution of RNA polymerase. The reconstitution of RNA polymerase was carried out by the method of Hager and Burgess (5). RNA polymerase (50 μg) was separated into subunits by SDS-polyacrylamide column gel (6-mm diameter) electrophoresis and stained with 0.25 M KCl containing 1 mM dithiothreitol, and then a piece of gel containing $\sigma^5$ was sliced and crushed in 300 μl of elution buffer. The eluted protein was precipitated with 1.2 ml of acetone, and the pellet was washed with 80% acetone to remove residual SDS. The pellet was dissolved in 5 μl of dilution buffer containing 6 M guanidine hydrochloride and then diluted with 250 μl of dilution buffer to reactivation. The renatured protein (15 μl) was mixed with 1 μg (1 μl) of vegetative core RNA polymerase which was purified by phosphocellulose chromatography (7), and the activity was assayed by runoff transcription.

Runoff transcription. The reaction mixture (45 μl) contained 50 mM Tris hydrochloride (pH 7.8), 5 mM magnesium acetate, 90 mM potassium acetate, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1 μg of DNA template, and an appropriate amount of RNA polymerase. The reaction mixture was incubated at $37^\circ$C for 10 min. RNA synthesis was initiated by the addition of a 5-μl mixture of 2 mM (each) ATP, GTP, and CTP-3.3 μCi of [α-32P]UTP (5,000 Ci/mmol). After 1 min, 1 μl of rifampin (0.5 mg/ml) was added to prevent reinitiation. The mixture was incubated at $37^\circ$C for 5 min, and then 1 μl of cold 10 mM UTP was added to the reaction mixture. After an additional 5 min, RNA was precipitated with 2 volumes of ethanol–0.3 M sodium acetate. The precipitate was dissolved in 20 μl of 60% formamide containing 0.1% bromophenol blue and then heat denatured (95°C, 5 min). The denatured sample (10 μl) was subjected to electrophoresis on a 5% polyacrylamide gel containing 7 M urea.

In vitro DNA-directed protein synthesis. In vitro DNA-directed protein synthesis was carried out as described previously (22). The reaction mixture (final volume, 50 μl) was modified slightly: 50 mM Tris hydrochloride (pH 7.8)–40 mM ammonium acetate–16 mM magnesium acetate–90 mM potassium acetate–2 mM dithiothreitol–5% (wt/wt) PEG 6000–45 μM folinic acid–6 mM ATP–0.5 mM (each) GTP, CTP, and UTP–20 mM phosphoenolpyruvate–9.1 μM [14C]leucine (342 μCi/μmol)–0.1 mM (each) amino acids (the other 19)–125 μg of PEG-treated S-30 purified from UOT0999 cells–1.5 A$_{260}$ units of preincubated ribosome–10 μg of B. subtilis tRNA purified from JH642 cells–1 μg of pUCVE-1 DNA was used as a template. After the reaction mixture was incubated at $37^\circ$C for 1 h, the samples were precipitated with trichloroacetic acid and dissolved in sample buffer (22). Samples were subjected to SDS-polyacrylamide (14% acrylamide) gel electrophoresis. After electrophoresis, the products were detected by fluorography (3).

RESULTS AND DISCUSSION

In vitro transcription of the spoVE gene. From the transcriptional spoVE-subtilisin gene fusion and the high-resolution primer extension mapping experiments, we showed that two transcriptional start sites, P1 and P2, are located 335 or 336 and 197 or 198 bp upstream from the NruI site, respectively (Fig. 1) (29). The nucleotide sequences of the −35 and...
and *spoVE* transcription activity was assayed by runoff transcription with the 1.3-kb *EcoRI-NruI* fragment (Fig. 1) containing *spoVE* promoters as a template. In the reaction with vegetative-cell RNA polymerase, no transcript was detected. The transcript was observed slightly when RNA polymerase prepared from *T*₀ cells was used and increased gradually until *T*₀ (Fig. 2B). The major transcripts were 340- and 180-base products (Fig. 2B and Fig. 3). These sizes agree with the in vivo data obtained by primer extension mapping analysis (29). Furthermore, when the 437-bp *Sau3AI* fragment (Fig. 1) which contains P2, but not P1, was used as a template, a 370-bp transcript was obtained in a runoff transcription experiment (Fig. 3). Since the *Sau3AI* site is located 175 bp downstream from the *NruI* site (27), the shift in size of the transcript from 180 to 370 bp indicates clearly that the location of P2 and the direction of transcription fit the prediction obtained from the in vivo experiments. These results indicate strongly that the *spoVE* gene has two promoters which are recognized by RNA polymerase present in the sporulating cells.

**Purification of RNA polymerase that transcribes the *spoVE* gene.** To determine the sigma factor which recognizes *spoVE* promoters, we purified RNA polymerase from *T*₀ cells by the

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**FIG. 2.** *spoVE*-transcribing activity of RNA polymerases purified by the small-scale method from cells of various stages. (A) SDS-polyacrylamide gel profile of RNA polymerases from vegetative (V) and *T*₀ (0), *T*₁ (1), *T*₂ (2), *T*₃ (3), *T*₄ (4), *T*₅ (5), and *T*₆ (6) cells. M, molecular size markers (sizes are indicated in kilodaltons). (B) Runoff transcription by 10 μl each of RNA polymerase fractions with the 1.3-kb *EcoRI-NruI* fragment as a template. After reaction at 37°C for 10 min, samples were subjected to 5% polyacrylamide gel (containing 7 M urea) electrophoresis. Products were detected by autoradiography. Markers were runoff transcripts of *Δ29 HindIII* digests (shown on the left side of panel B). Transcripts from P1 and P2 promoters are indicated.

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**FIG. 3.** Runoff transcription of the *EcoRI-NruI* and *Sau3AI* fragments. Fragments were transcribed by the *T*₁ RNA polymerase. Lanes: M, size markers which are 450- and 80-base RNAs transcribed from *Δ29 HindIII* fragments; 1, runoff transcript from the 1.3-kb *EcoRI-NruI* fragment; 2, runoff transcript from the 437-bp *Sau3AI* fragment.

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-10 regions of P2 were similar to those of the *spoIIA* gene, which is recognized by a sporulation-specific sigma factor, σ⁺ (26, 29). Similar sequences (Gaataaca [-35] and gATAaatgT [-10]) were found at the upstream region of P1 (29).

To analyze in vitro transcription of the *spoVE* gene, RNA polymerases from vegetative and sporulating cells of UOT0999 were purified by the small-scale method (Fig. 2A)
large-scale method described in Materials and Methods. This method has an additional purification step (HW 65F gel filtration) which is lacking in the small-scale method. Fractions of RNA polymerase eluted from a DNA-cellulose column with a linear salt gradient (0.25 to 1.5 M) were analyzed by SDS-polyacrylamide (12.5% acrylamide) gel electrophoresis (Fig. 4A) and assayed by runoff transcription with the spoVE promoter fragment (Fig. 4B). The transcript from the P2 promoter (180 bases) was detected, but that from the P1 promoter was not detected. The absence of P1 transcript suggests the possibility that the RNA polymerase which recognizes the P1 promoter may be associated with an additional low-molecular-weight factor(s) which was removed by the gel filtration process in the large-scale purification method. The transcriptional activity coincided with the presence of a protein of about 33 kDa in the purified RNA polymerase preparation. The size of the protein suggests that it may be a sigma factor, $\sigma^E$. However, the apparent molecular weight of this protein is larger than that of the $\sigma^E$ previously described (6, 21).

Amino acid sequence of the 33-kDa protein. Since the size of the 33-kDa protein is larger than the reported size (29 kDa) of $\sigma^E$ (6, 21), the N-terminal amino acid sequence of the 33-kDa protein was determined. RNA polymerase containing the 33-kDa protein was electrophoresed and blotted onto a polyvinylidene difluoride membrane. The stained 33-kDa protein band was cut out, and its N-terminal amino acid sequence was determined with the amino acid sequencer (Applied Biosystems model 477A). The N-terminal amino acid sequence of the 33-kDa protein was Tyr-Ile-Gly-Gly-Ser-Glu-Ala-Leu-Pro-Pro-Pro-Leu, which coincides with that of $\sigma^E$, except that the sequence was 2 amino acids longer than that reported by LaBell et al. (12). Probably pro-$\sigma^E$ (P$^{31}$) is cleaved first between Tyr-27 and Tyr-28 and then at Ile-29 and Gly-30. Apart from the processing site, it is clear that the sigma factor which recognizes the spoVE P2 promoter is $\sigma^E$.

Reconstitution of the spoVE-transcribing activity. To demonstrate that the 33-kDa protein is a sigma factor which recognizes the P2 promoter, we purified the 33-kDa protein by SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel was stained in the presence of 0.25 M KCl and the 33-kDa protein band was excised. The gel slice was
The spoVE gene is transcribed from P1 and P2 promoters and that the P2 promoter is recognized by $\sigma^E$-associated RNA polymerase. In a previous report (23), we concluded that spoVE gene expression is regulated at the translational level, since the SpoVE protein was synthesized specifically in the transcription-translation coupled system prepared from $T_2$ and $T_3$ cells but not in the coupled system prepared from vegetative cells, although the spoVE mRNA was detected in vegetative cells in vivo (23).

New data suggest that this interpretation is incorrect. Determination of the nucleotide sequence of the spoVE gene revealed that its product is similar to that of the E. coli ftsW gene (8), which is located in the mra region, a cell division gene cluster (18). We think that the B. subtilis spoVE region is a counterpart of the E. coli mra region, since the open reading frames of upstream (unpublished data) and downstream (19) regions of spoVE are similar to those of the E. coli ftsW gene (1, 9, 17). The spoVE region is expressed during vegetative growth (23), but we could not find the vegetative promoter in the 1.3-kb EcoRI-NruI fragment, suggesting that the vegetative promoter may lie in the region upstream of the 2.8-kb EcoRI fragment (30). This may be the reason why we could not detect SpoVE protein in the coupled system prepared from vegetative cells with a template which carries only the 2.8-kb EcoRI fragment. Then, what is the function of the P2 promoter? One possibility is that, in sporulating cells, more SpoVE protein may be required than that required in vegetative cells for the synthesis of sporulation-specific peptidoglycan in the spore septa (10) and cortex.

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REFERENCES


